ANNEX 2

Establishment of Duck cell line EB24 from duck ES cells

1 - RAW MATERIAL

Duck Eggs

Duck eggs from Peking strains GL30 were obtained from GRIMAUD FRERES SELECTION (La Corbière, Roussay France). The parent ducks were vaccinated against *Escherichia Coli* (Autogenous vaccine Coli 01 & 02), *Pasteurella multocida* (Landavax), Duck viral hepatitis (Hepatovax), *Erysipelothrix rhusiopathiae* (Ruvax), Avian metapneumovirus (Nemovac), *Salmonella typhimurium* & *Enteridis* (Autogenous vaccine), *Riemerella antipestifer* (Autovaccine Riemerella), Avian metapneumovirus (Nobilis RTV inactive) and *Erysipelothrix rhusiopathiae* (Ruvax). After receipt, fertilized Peking duck eggs were submitted to a disinfection in an hypochloryde bath followed by a decontamination with Fermacidal (Thermo) to avoid any risk of contamination linked to dusts attached on the shell.

Feeder cells

In the first step of the process, cells from murine origin (STO cells) were used as feeder layer to maintain the pluripotency of duck stem cells. Those feeder cells are mitotically inactivated by gamma irradiation (45 to 55 Grays) before seeding on plastic. This dose of irradiation is a sub-lethal dose that induces a definitive arrest of the cell cycle but still permits the production of growth factors and extracellular matrix, necessary for the promotion of the cell growth of non differentiated cells. The STO cell line was derived by A. Bernstein, Ontario Cancer Institute, Toronto, Canada from a continuous line of SIM (Sandos Inbred Mice) mouse embryonic fibroblasts and it was supplied by the American Type Culture Collection (ATCC) (STO Product number: CRL-1503, Batch number 1198713). Fresh feeder layers were prepared twice a week. Exponentially cells were dissociated and counted. A part of cells were seeded for maintenance of viable cultures and another part was irradiated. For irradiation, we prepared a cell suspension at $10x10^6$ cells/mL in tubes. Cells were exposed to a 45 to 55 grey dose and were seeded on plastic. After seeding, dishes or plates coated with inactivated feeder cells were used during a maximum of 5 days.

Medium

Medium EX-CELL ™ 65319, 63066 and 66444 (SAFC, customized medium)

Medium GTM-3 (Sigma, Cat n° G9916)

DMEM F12 (Cambrex, Cat n° BE04-687)

DMEM (Cambrex, Cat n° BE 12-614F)

Additives

Glutamine (Cambrex, Cat n° BE17-605E)

Pencillin/streptomycin (Cambrex, Cat n° BE17-602E))

Non essential Amino Acids (Cambrex, Cat n° BE13-114E)

Sodium pyruvate (Cambrex, Cat n°BE13-115)

Vitamines (Cambrex, Cat n° 13-607C)

Beta Mercapto Ethanol (Sigma, Cat n° M7522)

Yeastolate (SAFC, Cat n° 58902C)

Buffer and fixators

PBS 1X (Cambrex, Cat n° BE17-516F)

Cryoprotective agent

Dimethyl Sulfoxyde (DMSO) (Sigma, Cat n° D2650)

Factors

Six different recombinant factors were used:

- ❒ Recombinant Human Ciliary Neurotrophic Factor (CNTF) (Peprotech Inc, Cat n° 450-13)
- ❒ Recombinant Human Insulin Like Factor I (IGF1) (Peprotech Inc, Cat n° 100-11)
- ❒ Recombinant Human Interleukin 6 (IL6) (Peprotech Inc, Cat n° 200-06)
- ❒ Recombinant Human soluble Interleukin 6 receptor (sIL6r) (Peprotech Inc, Cat n° 200-06 R)
- ❒ Recombinant Human Stem Cell Factor (SCF) (Peprotech Inc, Cat n° 300-07)
- ❒ Recombinant Human basic Fibroblast Growth Factor (bFGF) (Peprotech Inc, Cat n° 100-18B)

All those factors, excepted IL6r, are produced in E. Coli bacteria. Soluble IL6r is expressed in transfected HEK293 cells.

2 - PROCESS OF ESTABLISHMENT OF DUCK CELL LINE EB24

Around 360 Fertilized duck eggs were opened, the yolk were separated from the albumen during the opening. The embryos were removed from the yolk with the aid of a small absorbent filter paper (Whatmann 3M paper), cut out beforehand in the form of a perforated ring with the aid of a punch. The diameter of the perforation is about 5 mm. These small rings were sterilized using dry heat for about 30 minutes in an oven. In practice, during the step of embryo collection, a small paper ring is deposited on the surface of the yolk and centered on the embryo which is thus surrounded by the paper ring. The latter is then cut out with the aid of small pairs of scissors and the whole removed is placed in a Petri dish, filled with PBS. The embryo thus carried away by the ring were cleaned of the excess yolk in the medium and the embryonic disk, thus free of the excess vitellin, were collected with a Pasteur pipette.

The duck embryos were placed in 50 mL tubes containing PBS 1X.The duck embryos are then mechanically dissociated and seeded on an inactivated layer of feeder STO cells into complete culture medium at 39°C, 7,5 % CO_2. The feeder cells were seeded in 6 well plates or dishes at around 2,7 $x10^4$ cell/cm^2. The complete culture medium is composed of serum free medium DMEM-Ham F12 supplemented with 10 % fetal bovine serum, with IGF1, CNTF, Il-6, Il-6R, SCF and FGF at a final concentration of 1ng/ml, and with 1 % non-essential amino acids, with 1 % of mixture of vitamins of commercial origin, with sodium pyruvate at a final concentration of 0,1 mM, with beta-mercapto-ethanol at a final concentration of 0.5 mM, glutamine at a final concentration of 2,1 mM, penicillin at a final concentration of 100 U/ml, streptomycin at a final concentration of 100 µg/ml and 1X yeastolate.

Rapidly after the first passages of the cells, the mixture of antibiotics is no longer added to the medium. The expression rapidly is understood to mean after the first 3 to 9 passages in general.

The duck ES cells are cultured in the DMEM-Ham F12 complete medium up to passage 7. After passage 7, the base medium is modified and DMEM-Ham F12 complete medium is replaced by the GTM-3 complete medium supplemented with 10 % fetal bovine serum, with IGF1, CNTF, Il-6, Il-6R, SCF and FGF at a final concentration of 1ng/ml, with 1 % non-essential amino acids, with 1 % of mixture of vitamins of commercial origin, with sodium pyruvate at a final concentration of 0,1 mM, with beta-mercapto-ethanol at a final concentration of 0.5 mM, glutamine at a final concentration of 2,1 mM, penicillin at a final concentration of 100 U/ml, streptomycin at a final concentration of 100 µg/ml and yeastolate 1X. Thus, at passage 11, the serum concentration is decreased at 5 % and SCF, IL6, IL6r and bFGF are removed for the medium. So, from passage 11, the medium is composed of 5 % FBS, with IGF1 and CNTF at a final concentration of 1ng/mL with 1 % non-essential amino acids, with 1 % of mixture of vitamins of commercial origin, with sodium pyruvate at a final concentration of 0,1 mM, with beta-mercapto-ethanol at a final concentration of 0.5 mM, glutamine at a final concentration of 2,1 mM, penicillin at a final concentration of 100 U/ml, streptomycin at a final concentration of 100 µg/ml and yeastolate 1X. A simultaneous withdrawal of IGF1 and CNTF is performed at passage 22. No recombinant factors are present in the GTM-3 culture medium after passage 22. Duck cells were maintained in a such medium between passage 23 and passage 28. When the duck ES cells from Pekin duck embryos are passaged from a culture dish to another, the seeding of culture dish was performed with around between 7 x 10^4/cm^2 to 12 x 10^4/cm^2 of duck ES cells in the complete culture medium. Preferably, the seeding is made with around 7.3 x 10^4/cm^2 (4 x 10^6 cells/55cm^2 or 4 x 10^6 cells/100 mm dish). Then, after passage 28, depletion of feeder cells is performed by a progressive decrease of feeder cells concentration over several passages. The dishes were originally seeded with around 2,7 x10^4 feeder cells/cm^2, then around 1,8 x 10^4 feeder cells/cm^2 between passage 29 and 33, then around 1,4 x10^4 cells/cm^2 between passage 34 and 37, then around 1 x 10^4 feeder cells/cm^2 between passage 38 and 42, then around 0,7 x 10^4 feeder cells/cm^2 between passage 43 and 46, and finally from passage 47 dishes were seeded only with avian cells and without feeder cells,. At the end of the feeder depletion, the dishes are seeded with 9 x 10^4 avian cells/cm^2 to 12,7 x 10^4 avian cells/cm^2. The depletion of feeder cells started at passage 29 and ended at passage 47. During the depletion of feeder cells, the duck ES cells are seeded in culture dishes at a higher concentration than in step a), about around 9 x 10^4 cell/cm^2 to 12,7 x 10^4 cell/cm^2. After several passages without feeder cells, growth parameters (Population Doubling Time (PDT) and Density) were studied to confirm cell stability and robustness and to initiate the cell growth as suspension. Cells are considered as enough robust to be submitted to a culture in suspension if, PDT is lower than around 40 hours and cell density higher than around 26 x 10^4 cells/cm^2. Moreover, cells morphology should be: round, refringent, very small and the cells shall not attached to the plastic dish too much. In the case of the EB24 cell development, culture in suspension is initiated at passage 48. 8 x10^6 cells were transferred in a Ultra Low attachment dish and maintained under constant agitation at around 50 to 70 rpm. For the next passages, cells were seeded in T175 flasks (Sarsted, ref 831812502) at a concentration

comprise between 0,4 to $0,5x10^6$ cells/mL. Following a short period of adaptation to the new conditions of culture, cell PDT decreased from around 248 H to 128 hours and the next step of deprivation is then performed. Thus at passage 52, vitamines, non essential amino acids, sodium pyruvate and beta mercaptethanol are removed. Regarding the good evolution of the PDT reaching 44 hours, at passage 56, from passage 57, the serum deprivation was initiated. Thus from passage 57, the serum free culture medium GTM-3 was supplemented with 5 % Foetal Bovine Serum (FBS), 1X yeastolate and 2,5 mM glutamine only. The serum depletion is performed on cell suspensions already depleted in growth factors, feeder cells, vitamins, non essential amino acids, sodium pyruvate and beta-mercaptoethanol. Serum depletion was performed by a progressive decreasing starting from 5 % serum, then 2.5 %, then 2 %, then 1.5 % of serum concentration in SFM cell culture medium to finally reach 0 % serum in SFM cell culture medium. Serum depletion started at passage 57 and ended at passage 77. During this serum depletion, adaptation to growth at 37°C was also performed. Thus at passage 65, cells growing in the culture medium supplemented with 2,5 % FBS were transferred at 37°C avoiding a progressive temperature shift. At the end of serum depletion, anchorage independent duck EB24 cells were able to grow at 37°C in absence of grow factors, in absence of feeder cells, in serum free medium.

After the obtaining of duck EB24 cells able to grow at 37°C in the SFM GTM-3 supplemented by 2,5 mM glutamine, some further adaptation were made by dilution or progressive adaptation in new SFM formulations as Excell 63066, Excell 66444, Excell CHO ACF from SIGMA-ALDRICH.

Figure 1: Electronic microscopy photograph of a Duck embryonic derived stem cell (EBx cell N° EB24)

Figure 1